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Short-Term Carcinogenesis and Mutagenesis Bioassays of Unregulated Automotive Emissions

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Evaluation of the potential risk of a chemical or environmental emission causing chronic health effects requires data from one or more of the following sources: epidemiologic and clinical studies of human exposure and effects; chronic (long term) bioassays in animals; and short-term bioassays in animals, animal and human cells, insects, plants, and microorganisms. Although it is advantageous to have data from as many different sources and bioassays systems as possible, human and chronic animal data are essential to the definitive determination of whether a substance poses a carcinogenic hazard to humans. Often, however, such data are not available for the evaluation of emissions from new or alternative technologies. In particular, data from long-term human or animal studies are rarely available before the introduction of new emission sources. Short-term tests, therefore, provide important suggestive evidence of a substance's potential to cause genotoxic effects. Scientific evidence continues to indicate that latent diseases, including cancer and genetic disease, may be initiated by alterations in the genetic DNA of a cell. Although the organization and amounts of DNA vary between organisms, the basic structure of DNA is the same. This fundamental similarity between the genetic material in all cells is the basis of short-term carcinogenesis and mutagenesis bioassays. Short-term tests use such microorganism as bacteria and yeast, plants, insects, isolated mammalian cells to detect.

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SHORT-TERM CARCINOGENESIS AND MUTAGENESIS BIOASSAYS OF UNREGULATED AUTOMOTIVE EMISSIONS*

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EVALUATION of the potential risk of a chemical or environmental emission causing chronic health effects requires data from one or more of the following sources: epidemiologic and clinical studies of human exposure and effects; chronic (long-term) bioassays in animals; and short-term bioassays in animals, animal and human cells, insects, plants, and microorganisms.

Although it is advantageous to have data from as many different sources and bioassays systems as possible, human and chronic animal data

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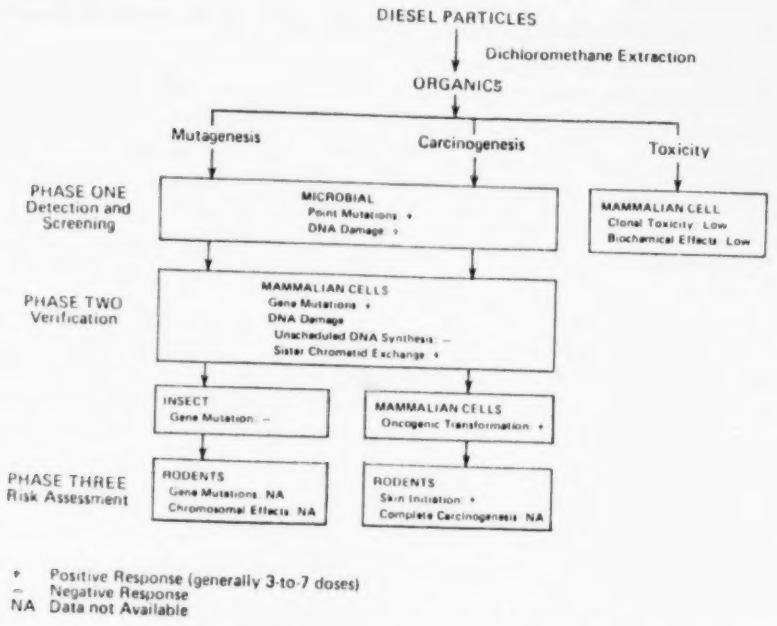
are essential to the definitive determination of whether a substance poses a carcinogenic hazard to humans.[1] Often, however, such data are not available for the evaluation of emissions from new or alternative technologies. In particular, data from long-term human or animal studies are rarely available before the introduction of new emission sources. Short-term tests, therefore, provide important suggestive evidence of a substance's potential to cause genotoxic effects.

Scientific evidence continues to indicate that latent diseases, including cancer and genetic disease, may be initiated by alterations in the genetic DNA of a cell. Although the organization and amounts of DNA vary between organisms, the basic structure of DNA is the same. This fundamental similarity between the genetic material in all cells is the basis of short-term carcinogenesis and mutagenesis bioassays. Short-term tests use such microorganisms as bacteria and yeast, plants, insects, isolated mammalian cells, and whole animals to detect a substance's genotoxicity or ability to alter the DNA.

A number of chemicals (promutagens or procarcinogens) require mammalian microsomal metabolism for conversion to mutagenic forms. Therefore, biological test systems, such as bacteria and certain mammalian cells which lack these microsomal enzymes, often use liver microsomal (S9) preparations as an activation mechanism. Chemicals that do not require this activation are considered direct-acting mutagens.

To evaluate a previously untested chemical or complex environmental pollutant, a three-step, phased approach to testing[2] is often the most time-, cost-, and information-effective. The figure illustrates the application of a phased approach to the assessment of diesel particles. Bioassays utilizing microorganisms are usually employed during phase one to provide detection or screening data. These results are tested for confirmation in phase two tests, which include mammalian cell, plant, insect, and short-term whole animal bioassays. Phase three tests include long-term animal and human studies. Specific bioassays to be included in each phase depend on a number of considerations, including the type of information desired, the nature of the sample, and the resources and time available. More than one test is included in each phase to detect different biological end points related to genotoxicity, including: gene mutations, DNA damage and repair, chromosomal effects, morphological oncogenic cell transformations, and tumor formation.



Application of a phased approach for the bioassay of diesel particle extracts. + = positive response (generally three-to-seven doses), – = negative response, NA = data not available.

Complex Mixtures

Industrial and energy processes generate gaseous, liquid, and solid waste streams composed of numerous chemical compounds and elements. These complex mixtures, including unregulated automotive emissions, may have dozens, hundreds, or thousands of individual components. Qualitative and quantitative identification of all of these individual components is a tremendous task, and the analytical challenge is easier if the number of compounds requiring identification can be reduced. For our purposes, this reduction should narrow compounds requiring identification to those potentially responsible for adverse health effects. The short-term bioassays used in the initial (phase one) screening of complex mixtures are useful to:

1) Indicate particular emissions or portions of an emission that are

potentially toxic, mutagenic, or carcinogenic and that should be evaluated in confirmatory and, possibly, long-term bioassays

2) Biologically direct the fractionation and identification of hazardous components and specific chemicals in complex mixtures

3) Compare the relative biological activity of similar emissions from alternative sources, fuels, control technologies, or operating conditions

Application of Phase One Screening Bioassays to Unregulated Automotive Emissions

Unregulated components of automotive emissions that have been receiving considerable attention are the complex mixtures of organics and particulate matter associated with incomplete combustion. Introduction of increasing numbers of light-duty diesel automobiles has stimulated environmental concern over the health effects of particulate emissions from these engines. Currently, diesel automobiles emit over 100 times the particles in grams per mile emitted by gasoline powered, catalyst equipped (gasoline catalyst) automobiles. Diesel particles emitted as carbonaceous soot serve as condensation nuclei for higher molecular weight organic combustion vapors. These vapors condense onto the soot particles as the exhaust is diluted and cooled to ambient temperature. The resulting diesel particles are emitted into the ambient air and contain 10 to 50% extractable organic constituents.

Gaseous organic compounds that do not adsorb onto particles are currently regulated only as total hydrocarbon emissions. This general class of emissions contains components, not specifically regulated, of potential concern, such as aldehydes, nitrosamines, phenols, and cyanides. Although research to apply short-term bioassays to these gaseous emissions is being initiated, most of the research completed to date has concentrated on the extractable organics from diluted particulate emissions.

The initial application of screening bioassays to automotive particulate emissions was undertaken to reduce the analytical task of identifying the hundreds of individual chemicals present in this complex mixture. Several hundred grams of diesel particles were collected after dilution from each of two heavy duty diesel engines.[3] Particle emissions from one of these engines (a Caterpillar 3208, four-stroke cycle engine) contained 24% extractable organic compounds. Extractable organic compounds from these particles were fractionated into acidic, basic, and neutral (paraffins, aromatics, and polar neutral) components prior to bioassay.

COMPARATIVE ACTIVITY RANKINGS* FOR MOBILE SOURCE MATRIX

Activity	*Heavy-duty diesel*	*Automotive emissions** Light-duty diesel*			*Gasoline-catalyst*
	Cat	*Nissan*	*Olds.*	*VW Rab.*	*Mustang*
Microbial mutation†	4.3	100	23	22	25
Sister chromotid exchange‡	0	100	0	50	I
Mammalian cell mutation‖	I	100	64	50	36
Rodent skin-tumor initiation§	0	100	45	I	35

*The Nissan diesel was given a value of 100 in order to normalize the activities to common units for comparison. All other data are expressed as a percentage of the Nissan activity.

**Cat is the Caterpillar 3208, 4-stroke cycle engine; Olds. is Oldsmobile; VW Rab. is Volkswagon Rabbit.

†*Salmonella typhimurium*, histidine reversion assay; data are shown for TA98 and S9 activation (arochlor-induced).

‡Chinese hamster ovary cell assay with arochlor-induced S9 activation.

‖L51784 mouse lymphoma, forward mutation assay at the thymidine kinase locus with arochlor-induced S9 activation.

§Sencar mouse assay using TPA as the tumor promoter.

I = data incomplete.

Bioassays used in initial screening of these organic fractions employed bacteria (*Salmonella typhimurium*) to detect gene mutations and mammalian cells to detect cellular toxicity. None of the diesel organic fractions were found highly toxic in mammalian cell assays, and all but one of the fractions demonstrated some mutagenicity in the *Salmonella typhimurium* plate incorporation assay for gene mutations.

Neutral components of diesel extract accounted for 84% of the mass and were fractionated into four subfractions (paraffins, aromatics, and transitional and oxygenated polar neutrals). The paraffinic fraction, 39% by weight, was not mutagenic; and the aromatic fraction, 13% by weight, accounted for only 1.5% of the mutagenic activity in the TA98 strain of *S. typhimurium*. The two polar neutral fractions, the transitional and oxygenated fractions, were the most mutagenic. These two fractions accounted for one third of the mass of the extractable organics and more than 90% of the mutagenic activity in both TA98 and TA1538 strains of *S. typhimurium*.

These tests and further studies suggest that more than one mutagen is present in the polar neutral fractions of organic compounds bound to diesel particles. These mutagens are primarily direct-acting, as evidenced

by the small effects of metabolic activation on the mutagenicity. Comparison of the mutagenic activity in various tester strains shows that activity in the tester strains responded to frameshift mutagens, for example, large planar molecules that intercalate in and cause a shift in the translation of the DNA molecule. Other studies show that these mutagens are not artifacts of the extraction or fractionation processes.[3]

Fractions of uncombusted fuel were not mutagenic, suggesting that the mutagens are products of the combustion process. Different fuels do, however, appear to influence the mutagenicity of the particle-bound, combustion organics. Studies comparing the mutagenic activity of combustion emission organics from two passenger cars operated with five different fuels showed that the poorest quality fuel (No. 2 diesel) generated the largest quantity of mutagenic, particle bound organics.[3] This minimum quality fuel had the lowest cetane index (41.8), highest aromatic content, and highest nitrogen and sulfur content.

While research continues to chemically identify and characterize the organic mutagens present on diesel particles, comparisons can be made of the effect of engine, fuel, and operating conditions on the mutagenicity of automotive emissions. Different engines, fuels, and operating conditions may affect not only the mutagenic activity of the organic fractions but the amount of extractable organic compounds present on the diesel particles and the particle emission rate. These factors can be accommodated by calculations to determine mutagenic activity on a per-mile or per-kilogram-fuel-consumed basis.

In comparing a series of different diesel automobiles, Claxton and Kohan[1] found as much as a threefold difference in the mutagenic emission rate. Although extractable organic compounds from the gasoline catalyst automobile emissions were more mutagenic than many of the diesel organic compounds, the amount of extractable organic compounds and the particle emission rate were so low for the gasoline catalyst automobile that the net mutagenic activity per mile was approximately two orders of magnitude (100 times) less than the comparable diesel automobile.

The total mutagenic activity resulting from automotive emissions depends on the release of mutagenic organic compounds from the particles. In laboratory tests organic solvents are used to remove mutagens from particles; studies comparing the effectiveness of different solvents and solvent systems have shown that dichloromethane extraction results in the most mutagenic extract.[5] The ability of physiological fluids (serum, lung

cell cytosol, and lung lavage fluid) to release mutagens from diesel particles has been compared to the extraction capability of solvents. Serum and lung cytosol were found to remove 80 to 85% of the solvent-extractable mutagenic activity from the diesel particles.[5] Serum- and cytosol-associated mutagens were essentially undetectable when the serum itself was tested in the *S. typhimurium* mutagenesis bioassay, possibly because of binding of mutagens by the serum. Other studies have shown that whole diesel particles are engulfed by mammalian cells *in vitro* and are capable of causing gene mutation.[6]

Utilizing the Phased Approach to Screen, Confirm, and Establish Priorities for Risk Assessment

Screening (phase one) bioassays are useful in identifying particulate emissions, or portions of an emission, that should be evaluated using higher level test systems (for example, mammalian cells and whole animals) and, possibly, long-term bioassays. Extractable organic compounds from diesel particles, although demonstrating low cellular toxicity, were mutagenic in a microbial (*S. typhimurium*) assay and positive in a yeast (*S. cerviciae*) assay for DNA damage (mitotic recombination). These results indicate that potentially mutagenic or carcinogenic chemicals are present in diesel emission organics.

Phase two bioassays were initiated to verify these screening results. Mammalian cells employed for phase two tests are more complex than microbial systems, and have cellular and chromosomal organization more similar to human cells than the cells used in phase one testing. Diesel organic compounds were positive in two gene mutation assays using mammalian cells. Two assays for DNA damage were also used: unscheduled DNA synthesis and sister chromatid exchange assays. The former assay was negative and the latter assay was positive for the diesel organic compounds tested.[7]

Phase two bioassays, in addition to verifying phase one results, delineate the biological end point or effect that should be investigated in long-term bioassays to assess risk. Two assays were selected to delineate the potential mutational and chromosomal effects of the diesel fractions: sex-linked recessive lethal mutational assay in insects (mutational effect), and morphological oncogenic transformation in mammalian cells (chromosomal effect).

The mutational assay was conducted only for the most polar neutral (oxygenated) fraction from the heavy-duty diesel engine particulate emissions. Difficulties were encountered in administering the organic fraction, but toxicity was observed at the highest dose, and bioassay results were negative. The carcinogenesis assay for morphological oncogenic transformation was positive in the mammalian (BALB/c 3T3) cells, results that strongly suggest that long-term carcinogenesis bioassay research should be initiated. Such studies were begun by the Environmental Protection Agency in 1978 with both diesel organic compounds and diesel particle emissions.[8] Initial results of those studies show that diesel organic compounds are tumorogenic in rodents.[9,10]

Additional research is needed to determine which bioassays are most useful in evaluating automotive emissions and to develop new methods to expose these test systems to such difficult samples as gases and insoluble organics.

Comparative Mutagenic and Carcinogenic Activity of Unregulated Automotive Emissions

The phased approach described above is a time- and cost-effective way rapidly to identify emissions that should be evaluated by long-term bioassays. In addition, tests are generally conducted using three to seven doses so that quantitative dose-response data are usually obtained with a positive response. Evaluation of different emissions and emission sources has, therefore, yielded information on quantitative differences and similarities between the mutagenic activities of emissions from different sources. Such results indicate either the presence of different amounts of the same mutagen in the samples or, possibly, the detection of different mutagens. These quantitative comparisons may prove useful in comparing the potential hazard associated with different sources, fractions, fuels, or control devices. Evaluation of the correlation between these short-term bioassays and chronic (long-term) bioassays is required to estimate the potential human health hazard.

Several retrospective evaluations of *in vitro* and *in vivo* correlations for the assays discussed here are in progress for a series of pure compounds.[11,12] Much less is known about the quantitative correlation between results of bacterial, mammalian cell, and rodent carcinogenesis and mutagenesis assays involving complex mixtures. To determine quantitative correlations for complex emission products, simultaneous comparative

studies must be conducted using the same samples because these products may vary with operational and other characteristics. A matrix of *in vitro* and *in vivo* bioassays is currently used quantitatively to compare the biological activity of a series of unregulated emissions (extractable organic compounds from particulate emissions),[13] and some of the findings are summarized below.

Quantitative bioassys were performed on organic extracts from particulate emissions from the following series of automotive emission sources: light-duty Oldsmobile diesel 350 vehicle, heavy-duty Caterpillar diesel engine, light-duty Nissan diesel engine, Volkswagen Rabbit diesel vehicle, and a gasoline-catalyst Mustang vehicle.

The test matrix included the following bioassays: reverse mutation in *Salmonella typhimurium;* sister chromatid exchange in Chinese hamster ovary cells; gene mutation in L51784 mouse lymphoma cells, and skin-tumor initiation in Sencar mice.

Initial data from the comparative study of automotive emission organic compounds are shown as normalized rankings in the table. These bioassays were performed in the presence of exogenous metabolic activation (S9). The quantitative results from the mobile source samples show a general overall consistency. Organic compounds from the Caterpillar sample resulted in the least biological activity in all four bioassays and the Nissan sample showed the highest activity in all assays. The other three samples (Oldsmobile, Volkswagen, and Mustang) generally showed intermediate activity.

In theory, gene mutation and skin-tumor initiation arise from similar mechanisms and should thus give similar results, assuming equal toxicity and mutagen/carcinogen transport/activation by the various cell types. A comparison of the results of the microbial and mammalian cell mutation bioassays with the results of the rodent skin tumor initiation bioassay for the mobile source samples seems to support this hypothesis.

Other comparative source samples (roofing tar, coke oven, and cigarette smoke condensate) evaluated in this study showed less agreement between quantitative results from these bioassays and other bioassays.[14] Thus, it may not be possible quantitatively to extrapolate from *in vitro* results to *in vivo* effects for all types of complex mixtures.

SUMMARY

Short-term carcinogenesis and mutagenesis bioassays are now being

applied to the evaluation and characterization of unregulated automotive emissions. Standard test procedures are generally employed to filter emission particles from diluted vehicle exhaust. Organic compounds associated with both diesel and gasoline particle emissions exhibit mutagenic and carcinogenic activity in most short-term bioassays. The relative potency between different mobile sources varies significantly.

Current research focuses on the following areas: (1) comparative potency of emissions from a variety of mobile sources, (2) comparative evaluation of a battery of bioassays for mobile source applications, (3) identification of the hazardous components in diesel emissions, and (4) determination of the effective doses and targets for those hazardous components.

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